Prevention of selenite-induced cataractogenesis in Wistar rats by the polyphenol, ellagic acid

M. Sakthivel a, R. Elanchezhian a, E. Ramesh a, M. Isai a, C. Nelson Jesudasan b, P.A. Thomas b, P. Geraldine a,*

a Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli, 620 024 Tamil Nadu, India
b Institute of Ophthalmology, Joseph Eye Hospital, Tiruchirappalli, 620 001 Tamil Nadu, India

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Abstract

The present study sought to evaluate the efficacy of the naturally-occurring polyphenol, ellagic acid, in preventing selenite-induced cataractogenesis. In the present study, Wistar rat pups were divided into 3 groups of 15 each. Group I (normal) rats received an intraperitoneal (i.p.) injection of normal saline on postpartum day 10; group II (cataract-untreated) rats received a single subcutaneous (s.c.) injection of sodium selenite (19 μmol/kg body weight) on postpartum day 10; group III (cataract-treated) pups received a single s.c. injection of sodium selenite on postpartum day 10 and intraperitoneal injections of ellagic acid (200 mg/kg body weight) on postpartum days 9–14. At the end of the study period (30th postpartum day), slit-lamp examination of both eyes of each rat pup revealed no lenticular opacification (cataract stage 0) in all eyes of group I pups, definite nuclear cataracts (stages 4–6) in the eyes of all (100%) group II rat pups and no lenticular opacification in eight (53%) and mild lenticular opacification (cataract stages 1–3) in seven (47%) of group III rats (changes in group II vs group III, P < 0.01). The mean activities of the antioxidant enzymes catalase, glutathione peroxidase, superoxide dismutase and glutathione-S-transferase were significantly lower in lenses of Group II rats than in Group I or Group III rat lenses. In addition, the mean levels of GSH in lenses and erythrocytes were also significantly lower in Group II rats than in Group I or Group III rats. Conversely, the mean concentration of MDA (an indicator of lipid peroxidation) in lenses and erythrocytes was found to be significantly higher in Group II rats than that in Group I or Group III rats. Also, the mean concentration of calcium was found to be significantly higher in lenses of Group II rats than in those of Group I and Group III rats. The results suggest that ellagic acid can prevent or retard experimental selenite-induced cataractogenesis in Wistar rats. This protective effect in rat lenses appears to occur by maintaining the antioxidant defense system and inhibition of lipid peroxidation.

Keywords: oxidative stress; cataractogenesis; calcium; antioxidant; ellagic acid; selenite cataract

1. Introduction

Cataract refers to the opacification of the lens of the eye. In humans, bilateral cataract formation accounts for 42% of all blindness worldwide (Vision Research, 1998). Since there is presently no universally-accepted pharmacological agent that either prevents or reduces the opacification of the human lens, surgical removal is generally accepted as the only treatment for human cataract (Dogany et al., 2006). However, the cost of cataract surgery may place it beyond the reach of less affluent individuals. Hence, there is an urgent need for inexpensive, non-surgical approaches to the treatment of cataract (Olson et al., 2003).

Cataract is a multifactorial disease associated with several risk factors such as ageing, diabetes, malnutrition, diarrhoea, poverty, sunlight, smoking, hypertension, and renal failure (Harding, 1991). Free radical-induced oxidative stress is postulated to be perhaps the major factor leading to senile cataract formation (Gerster, 1989). This hypothesis is supported by the anticataractogenic effect of various nutritional (Gerster, 1989)...
and physiological (Devamanoharan et al., 1999; Yagci et al., 2006) antioxidants in experimental animals.

Selenium cataract is a rapidly-induced, convenient model for the study of senile nuclear cataractogenesis. The morphological and biochemical characteristics of this model have been extensively investigated; moreover, this model shows a number of general similarities to human cataract. The reliability and extensive characterization of selenium cataract makes it a useful rodent model for rapid screening of potential anticalaract agents (Shearer et al., 1997). Various natural and synthetic compounds of differing chemical structures have been reported to prevent selenium-induced cataract in vitro as well as in vivo (Tamada et al., 2001; Gupta et al., 2003, 2005; Doganay et al., 2006; Geraldine et al., 2006) by virtue of antioxidant properties. However, there is a need to evaluate additional compounds for their anticalaractogenic effects.

Ellagic acid (EA) is a polyphenolic compound found in nature in a wide variety of fruits and nuts such as raspberries, pomegranate, walnuts, grapes and blackcurrants. Ellagic acid has been found to possess antioxidant (Priyadarsini et al., 2002), antimutagenic (Kaur et al., 1997), and anti-inflammatory (Iltanola-Vormisto et al., 1997) properties, to scavenge both oxygen and hydroxyl radicals, and to inhibit lipid peroxidation and 8-OhdG formation in vitro and in vivo (Cozzi et al., 1995; Takagi et al., 1995; Laranjinha et al., 1996; Iino et al., 2001). In view of the antioxidant properties of ellagic acid, and since oxidative stress has been implicated in cataractogenesis, we reasoned that ellagic acid might exhibit antigalactogenic potential. This hypothesis was evaluated in vivo (in an experimental animal model) and also by estimating the antioxidant profile of lenses of rats treated with ellagic acid.

2. Materials and methods

2.1. Treatment protocol

Nine day-old rat pups (Wistar strain) were used in this study. The pups were housed with parents in large spacious cages, and the parents were given food and water ad libitum. The animal room was well-ventilated and had a regular 12:12-h light/dark cycle throughout the experimental period. These animals were used in accordance with institutional guidelines and with the Association for Research in Vision and Ophthalmology statement for the use of animals in research. The rat pups were divided into one control group and two experimental groups, each comprising 15 pups. In group I (control), saline was injected intraperitoneally on postpartum day 10. In both experimental groups (II & III), sodium selenite (19 μmol/kg body weight) was injected subcutaneously on postpartum day 10. In addition, pups in group III received intraperitoneal injections (200 mg/kg body weight) of ellagic acid (HiMedia Laboratories, India; Assay 95%); the first dose of ellagic acid was administered 1 day prior to the selenite injection (that is, on postpartum day 9), and was repeated once daily for five consecutive days thereafter (on days 10 through 14). The injections were given in the morning hours. On day 10 alone, Group III pups received ellagic acid one hour prior to selenite injection.

2.2. Morphological assessment of cataract

The development of cataract in the rat eyes was assessed once a week for 3 weeks after selenite injection by slit-lamp biomicroscopy. Prior to each examination, mydriasis was achieved in each eye by instilling one drop of a topical ophthalmic solution containing tropicamide with phenylephrine (Maxdal Plus, Hi-Care Pharma, Chennai, India) every 30 min while keeping the rats in a dark room for 2 h. The eyes were viewed under a slit-lamp biomicroscope at 12 x magnification; the observer did not know the identity of the animals before scoring the cataracts. At the final examination (postnatal day 30) any cataracts that had developed were graded (Hiroaka and Clark, 1995) and photographed.

The degree of opacification was graded as follows:

Stage 0—normal transparent lens.
Stage 1—lens with initial signs of a posterior subcapsular or nuclear opacity involving tiny scatters.
Stage 2—lens with a minimal nuclear opacity with swollen fibers or posterior subcapsular scatterings.
Stage 3—lens with a diffuse nuclear opacity.
Stage 4—lens with a partial nuclear opacity.
Stage 5—lens with a nuclear opacity not involving the lens cortex.
Stage 6—lens with a mature dense opacity involving the entire lens.

Following the final morphological examination at postpartum day 30, the animals were sacrificed by cervical dislocation; the lenses were at once dissected out for various biochemical studies. A sample of blood was also drawn from rat just before sacrifice, in order to investigate certain erythrocyte parameters; each blood sample was collected in a heparinized polypropylene tube.

The paired lenses from each individual rat were pooled together and considered as one individual unit when estimating the various values.

2.3. Biochemical analysis

2.3.1. Antioxidant enzymes

The lenses from each group of rats were homogenized in 10 times their mass of 50 mM phosphate buffer (pH 7.2), and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant obtained was stored at −70 °C in aliquots until used for the analysis of enzyme activities. To calculate the specific enzyme activity, protein in each sample was estimated by the method of Bradford (1976) by using bovine serum albumin as a standard.

2.3.1.1. Catalase (CAT). CAT activity was determined by the method of Sinha (1972). In this test, dichromic acetic acid is reduced to chromic acetate when heated in the presence of
hydrogen peroxide (H$_2$O$_2$), with the formation of perchloric acid as an unstable intermediate. In the test, the green colour developed was read at 590 nm against blank on a spectrophotometer. The activity of catalase was expressed as units/mg protein (one unit was the amount of enzyme that utilized 1 mmole of H$_2$O$_2$/min).

2.3.1.2. Glutathione peroxidase (Gpx). The activity of Gpx was determined essentially as described by Rotruck et al. (1973). The principle of this method is that the rate of glutathione oxidation by H$_2$O$_2$, as catalysed by the Gpx present in the supernatant, is determined; the colour that develops is read against a reagent blank at 412 nm on a spectrophotometer. In the test, the enzyme activity was expressed as units/mg protein (one unit being the amount of enzyme that converted 1 µmole of GSH to the oxidized form of glutathione [GSSH] in the presence of H$_2$O$_2$/min).

2.3.1.3. Superoxide dismutase (SOD). SOD activity was determined by the method of Marklund and Marklund (1974). In this test, the degree of inhibition of pyrogallol auto-oxidation by supernatant of the lens homogenate was measured. The change in absorbance was read at 470 nm against blank every minute for 3 min on a spectrophotometer. The enzyme activity was expressed as units/mg protein.

2.3.1.4. Glutathione-S-transferase (GST). The activity of GST was determined by the method of Habig and Jacoby (1981). The conjugation of GSH with 1 chloro, 2-4 dinitrobenzene (CDNB), a hydrophilic substrate, was observed spectrophotometrically at 340 nm to measure the activity of GST. One unit of GST was defined as the amount of enzyme required to conjugate 1 µmol of CDNB with GSH/min.

2.3.1.5. Glutathione reductase (GR). Glutathione reductase, which utilizes NADPH to convert oxidized glutathione to the reduced form, was assayed by the method of Stall et al. (1969). The change in absorbance was read at 340 nm for 2 min at intervals of 30 s in a UV-spectrophotometer. The activity of glutathione reductase was expressed as nmoles of NADPH oxidized/min/mg protein.

2.3.2. Estimation of reduced glutathione (GSH) and malondialdehyde (MDA) content in erythrocytes and lenses

Each blood sample collected was centrifuged (3000 × g, 4 °C, 5 min) to allow separation of the erythrocytes from the other blood cells. These erythrocytes were washed twice with equal volumes of 0.9% sodium chloride solution and then hemolyzed with twofold volumes of cold distilled water. A supernatant was obtained after centrifugation at 5000 × g for 30 min; the levels of GSH and MDA in the supernatant were then measured. Sample preparation was performed at 4 °C and sample storage was at −70 °C until analysis.

The GSH content was estimated by the method of Moron et al. (1979). Each lens was homogenized in 1 ml of 0.1 M phosphate buffer, and was centrifuged at 5000 rpm for 15 min at 4 °C. To the supernatant of the hemolysate and the supernatant of the lens homogenate, 0.5 ml of 10% trichloroacetic acid was added followed by recentrifugation. To the protein-free supernatant, 4 ml of 0.3 M Na$_2$HPO$_4$ (pH 8.0) and 0.5 ml of 0.04% (wt/vol) 5,5-dithiobis-2-nitrobenzoic acid were added. The absorbance of the resulting yellow color was read spectrophotometrically at 412 nm. A parallel standard was also maintained. The results were expressed in µmoles/g wet weight for the lenses and nmoles/g Hb for erythrocytes.

The extent of lipid peroxidation was determined by the method of Ohkawa et al. (1979). Briefly, 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.81% thiobarbituric acid aqueous solution were added in succession. To this reaction mixture, 0.2 ml of the tissue sample (hemolysate or lens homogenate) was added. The mixture was then heated in boiling water for 60 min. After cooling to room temperature, 5 ml of butanol: pyridine (15:1 v/v) solution were added. The mixture was then centrifuged at 5000 rpm for 15 min. The upper organic layer was separated, and the intensity of the resulting pink colour was read at 532 nm. Tetramethoxypropane was used as an external standard. The level of lipid peroxide was expressed as nmoles of MDA formed/g wet weight for lenses and nmoles of MDA formed/g Hb for erythrocytes.

2.3.3. Determination of calcium concentration in the lens

For this experiment, rats belonging to one stage of each experimental group was considered. Thus, the lenses from Group I (stage 0), Group II (stage 6) and Group III (stage 0) rats were subjected to Ca$^{2+}$ analysis. The dry weight of the lens was measured after heating at 100 °C for 20 h. The lenses were then digested with 0.2 ml concentrated HCl at room temperature overnight and adjusted to 1.0 ml with deionized water. The mixtures were centrifuged at 10,000 × g for 10 min to remove insoluble material, if any. The calcium concentrations in the supernatant fractions were then measured by an atomic absorption spectrophotometer (model Spectra AA-220, Varian), operated with a slit width of 0.5 nm, with the wavelength set at 422.7 nm. Standard solutions were prepared from CaCO$_3$ and deionized water. The results were expressed as µmoles of Ca/gm dry weight of the lens.

2.4. Statistical analysis

The mean value of each parameter in each individual group of rats was calculated from at least five individual values and was expressed as mean ± SD. Statistical analysis was done by using the Student’s t-test, chi-square test, correlation and linear regression. P values <0.05 were considered significant.

3. Results

3.1. Morphological assessment of changes in lenses

At the final examination on postpartum day 30, the pups were evaluated for cataract development and photographed. None of the group I (control) rats, which had received an
intraperitoneal (i.p.) injection of normal saline on postpartum day 10, developed cataractous lenses (Fig. 1). In group II, all (100%) rats, which had received a single subcutaneous (s.c.) injection of sodium selenite (19 μmol/kg body weight) on postpartum day 10, developed cataracts that were graded as being between Stage 4 and Stage 6 (Figs. 2–4). However in group III, only 7 of 15 (47%) rats, which had received a single s.c. injection of sodium selenite on postpartum day 10 and intraperitoneal injections of ellagic acid (200 mg/kg body weight) on postpartum days 9–14, developed cataracts that were graded as being between stage 1 and stage 3 (Figs. 5–7) (Table 1); this difference was statistically significant (χ² [df = 1] = 10.9; P < 0.01). The remaining 8 of 15 (53%) rats of group III had clear, normal lenses.

3.2. Effect of ellagic acid on antioxidant enzymes

3.2.1. Catalase

The mean activity of CAT (expressed as mmol H₂O₂ consumed/min/mg protein) in lenses of Group II rats (3.86 ± 0.2) was significantly (P < 0.001) lower than that in lenses of Group I rats (7.4 ± 0.2) and was also significantly (P < 0.001) lower than that in Group III rats (6.28 ± 0.6) (Table 2). However, the mean catalase activity in the lenses of Group III rats was also significantly (P < 0.05) lower than that in Group I rat lenses (Table 2). The catalase activity gradually decreased with increase in the stage of opacity (i.e., with increasing opacification) in the lenses of group II (Y = 5.40 – 0.77X, r = −0.99; df = 13) and group III (Y = 7.92 – 0.72X, r = −0.98; df = 18) rats.

3.2.2. Glutathione peroxidase

The mean activity of Gpx enzyme (expressed as μmol glutathione oxidized/min/mg protein) was significantly (P < 0.001) less in Group II (24.89 ± 1.2) rat lenses than that in Group I (34.17 ± 2.0) and Group III (29.79 ± 0.8) rat lenses (Table 2). While the mean activity of Gpx in lenses of Group III rats was significantly better (higher) than that in lenses of Group II rats, it was also significantly (P < 0.001) less than that in lenses of Group I rats (Table 2).

The activity of Gpx decreased gradually with increase in the stage of opacity (i.e., with increasing opacification) in the lenses of group II (Y = 26.85 – 1.03X, r = −0.99; df = 13) and Group III (Y = 31.74 – 0.78X, r = −0.99; df = 18) rats.

3.2.3. Superoxide dismutase

The mean activity of SOD (expressed as units/min/mg protein) in Group II rat lenses (1.0 ± 0.1) was significantly lower than that in lenses of Group I (2.29 ± 0.3) rats (P < 0.01) and Group III (1.71 ± 0.1) rats (P < 0.05) (Table 2). However, no significant differences were observed between the mean SOD activities in Group III and Group I rat lenses (Table 2). The SOD activity gradually decreased with increase in the stage of opacity (i.e., with increasing}

![Fig. 2. Stage 4 cataract: Lens with a partial nuclear opacity observed on postpartum day 30. Seen in 33% of Group II rats that had received a single subcutaneous injection of sodium selenite on postpartum day 10.](image)

![Fig. 3. Stage 5 cataract: Nuclear opacity observed on postpartum day 30. Seen in 40% of Group II rats that had received a single subcutaneous injection of sodium selenite on postpartum day 10.](image)
opacification) in the lenses of group II ($Y = 1.15 - 0.077X$, $r = -0.99$; df = 13) and group III ($Y = 1.88 - 0.068X$, $r = -0.99$; df = 18) rats.

3.2.4. Glutathione-S-transferase

The mean activity of GST (expressed as μmol of CDNB conjugate with GSH/min) was significantly reduced in group II (3.02 ± 0.4) rat lenses in comparison to that of lenses of group I (5.49 ± 0.7) and group III (4.44 ± 0.5) rats (Group II vs Group I, $P < 0.001$; Group II vs Group III, $P < 0.01$) (Table 2). However, no significant difference was observed between the mean GST activity in Group III and Group I rat lenses (Table 2). The GST activity gradually decreased with increase in the stage of opacity (i.e., with increasing opacification) in the lenses of group II ($Y = 3.57 - 0.28X$, $r = -0.99$; df = 13) and group III ($Y = 5.11 - 0.27X$, $r = -0.98$; df = 18) rats.

3.2.5. Glutathione reductase

The mean activity of GR (expressed as nmol of NADPH oxidized/min/mg protein) was significantly lower ($P < 0.01$) in group II rat lenses (0.23 ± 0.01) than that in group I rat lenses (0.29 ± 0.03). However, no significant differences were observed between the mean activity of GR in lenses of Group II and Group III rats (0.27 ± 0.04), and also between that of Group III and Group I rat lenses (Table 2). The activity of GR decreased gradually with increase in the stage of opacity (i.e., with increasing opacification) in the lenses of group II ($Y = 0.266 - 0.018X$, $r = -0.99$; df = 13) and group III ($Y = 0.286 - 0.007X$, $r = -0.99$; df = 18) rats.

3.3. Effect on GSH and MDA levels in erythrocytes and lenses

The mean GSH levels in erythrocytes (444.6 ± 59) and lenses (5.06 ± 0.6) of group II rats were significantly
(P < 0.001) lower than the levels in both group I [erythrocytes (784.7 ± 83) and lens (8.36 ± 0.4)] and Group III [erythrocytes (682.6 ± 71) and lens (7.53 ± 0.4)] rats (Table 3). However, significant differences were also observed in levels of GSH in erythrocytes (P < 0.001) and lenses (P < 0.05) between group III and group I rats (Table 3). The erythrocyte GSH level gradually decreased with increase in the lens opacity in group II (Y = 519 - 41.4X, r = -0.98; df = 13) and group III (Y = 759 - 30.7X, r = -0.99; df = 18) rats. So also the lens GSH level decreased gradually with increase in the stages of lens opacity (that is, with increasing opacification) in group II (Y = 5.68 - 0.31X, r = -0.99; df = 13) and group III (Y = 7.93 - 0.17X, r = 0.97; df = 18) rats.

The mean concentrations of MDA in erythrocytes (384.2 ± 46) and lenses (92.84 ± 4) of group II rats were significantly (P < 0.001) higher than the concentrations in group I [erythrocytes (285.4 ± 71) and lens (60.13 ± 3)] and Group III (erythrocytes (334.4 ± 75) and lens (76.24 ± 5)] rats (Table 4). However, there was also a significant (P < 0.001) difference in mean concentrations of MDA in erythrocytes and lenses between group III and group I rats (Table 4). On the other hand, the concentration of MDA in erythrocytes gradually increased with increase in the lens opacity in group II (Y = 366 + 9.19X, r = 0.99; df = 13) and group III (Y = 277.4 + 22.71X, r = 0.98; df = 18) rats. A similar trend of elevated lens MDA level with increased opacification was recorded in group II (Y = 87.37 + 2.73X, r = 0.99; df = 13) and group III (Y = 70.7 + 3.24X, r = 0.99; df = 18) rats.

### 3.4. Calcium levels in lenses

The calcium levels in the lenses of the three groups of rats are depicted in Fig. 8. The calcium concentration (1.21 ± 0.06) was significantly (P < 0.001) higher in the lenses of rats that had received selenite alone (Group II rats) than in those of ellagic acid-treated Group III animals (0.77 ± 0.04) and normal (Group I) rats (0.61 ± 0.01).

### 4. Discussion

Although cataract is the most prevalent condition leading to visual impairment, there is currently no routine pharmacological treatment for this condition. Ideally, a medicament for

### Table 2

Activities of antioxidant enzymes in lenses removed from rat pups of Group I, Group II and Group III.

<table>
<thead>
<tr>
<th>Enzymes studied</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase (mmol H₂O₂ consumed/min/mg protein)</td>
<td>7.40 ± 0.2</td>
<td>3.86 ± 0.2²</td>
<td>6.28 ± 0.6⁴</td>
</tr>
<tr>
<td>Glutathione peroxidase (μmol glutathione oxidized/min/mg protein)</td>
<td>34.17 ± 2.0</td>
<td>24.89 ± 1.2²</td>
<td>29.79 ± 0.8⁴</td>
</tr>
<tr>
<td>Superoxide dismutase (units/min/mg protein)</td>
<td>2.29 ± 0.3</td>
<td>1.0 ± 0.1⁴²</td>
<td>1.71 ± 0.1⁴</td>
</tr>
<tr>
<td>Glutathione-S-transferase (μmol of CDNB conjugate with GSH/min)</td>
<td>5.49 ± 0.7</td>
<td>3.02 ± 0.4⁴²</td>
<td>4.44 ± 0.5⁵⁴²</td>
</tr>
<tr>
<td>Glutathione reductase (mmoles of NADPH oxidized/min/mg protein)</td>
<td>0.29 ± 0.03</td>
<td>0.23 ± 0.01⁴²</td>
<td>0.27 ± 0.04⁵⁴²</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SD of five determinations. Group I: Normal. Group II: Animals exposed to selenite only. Group III: Animals exposed to selenite and treated with ellagic acid. N.S. Value not significantly different from group I value.

² Statistically significant difference (P < 0.001) when compared with group I and group III values.

³ Statistically significant difference (P < 0.05) when compared with group I value.

⁴ Statistically significant difference (P < 0.001) when compared with group I value.

⁵ Statistically significant difference (P < 0.01) when compared with group I value.

⁶ Statistically significant difference (P < 0.05) when compared with group III value.

### Table 3

Mean GSH level in erythrocytes and lenses.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH in erythrocytes* (μmoles/g Hb)</th>
<th>GSH in lenses* (μmoles/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>784.7 ± 83</td>
<td>8.36 ± 0.4</td>
</tr>
<tr>
<td>Group II</td>
<td>444.6 ± 59</td>
<td>5.06 ± 0.6²</td>
</tr>
<tr>
<td>Group III</td>
<td>682.6 ± 71b</td>
<td>7.53 ± 0.4²</td>
</tr>
</tbody>
</table>

* All values are expressed as mean ± SD of five determinations. Group I: Normal. Group II: Animals exposed to selenite only. Group III: Animals exposed to selenite and treated with ellagic acid.

² Statistically significant difference (P < 0.001) when compared with both group I and group III values.

### Table 4

Mean MDA level in erythrocytes and lenses.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA in erythrocytes* (μmoles/g Hb)</th>
<th>MDA in lenses* (μmoles/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>285.4 ± 71</td>
<td>60.13 ± 3</td>
</tr>
<tr>
<td>Group II</td>
<td>384.2 ± 46</td>
<td>92.84 ± 4²</td>
</tr>
<tr>
<td>Group III</td>
<td>354.4 ± 75b</td>
<td>76.24 ± 5b</td>
</tr>
</tbody>
</table>

* All values are expressed as mean ± SD of five determinations. Group I: Normal. Group II: Animals exposed to selenite only. Group III: Animals exposed to selenite and treated with ellagic acid.

² Statistically significant difference (P < 0.01) when compared with both group I and group III values.
cataarct should prevent or delay cataractogenesis and should also be able to cause regression factors of established catarac-tous changes. A key impediment to the formulation of an ideal anti-cataract medication is perhaps, the continued imperfect understanding of the mechanisms driving cataractogenesis.

Various experimental models have been developed to delineate the mechanism of cataractogenesis and to identify crucial targets in the process. The selenite cataract is an extremely rapidly-induced and convenient model of cataractogenesis. It is a useful in vivo rodent model for initial drug testing, which explains why a number of potential anti-cataract agents have been evaluated in the selenite cataract model (Shearer et al., 1997). However, it is important to remember that certain differences also exist between human and selenite cataracts, hence caution must be exercised when assessing the results obtained in studies on selenite cataract.

Although various biochemical changes associated with selenite-induced cataract have been reported, the mode of action of selenite is not completely understood. It is hypothesized that early changes in the lens epithelium may result from oxidative damage caused by selenite, possibly to critical sulph-hydryl groups on molecules such as Ca-ATPase or ion channels (Shearer et al., 1987). A progressive decline in transport of cations and activity of Na⁺, K⁺-ATPase has also been reported in lens membranes following exposure to selenite; these effects of selenite are partially reversed by high concentrations of GSH, suggesting an oxidative mechanism (Hightower and McCready, 1994). In the present study, the mean levels of calcium was found to be significantly higher in lenses of group II (selenite administered) rats than that in group I (normal) rats. These results are consistent with those of other workers (Hightower et al., 1980; David and Shearer, 1984). However, administration of ellagic acid to selenite-challenged pups significantly reduced the mean calcium level in group III rats. The increased calcium levels in the lenses of rats administered selenite may have been caused by oxidation of sulphhydryls and other changes to the membranes caused by selenite, leading to inhibition of the Na⁺, K⁺ and Ca-ATPase pump leading to influx of calcium. In rodent lenses, the influx of calcium then causes activation of the calcium-dependent pro-teases calpain II and Lp82, which partially degrade α and β crystallins, ultimately causing insolubilization of protein and scattering of light. This is accompanied by a decrease in the activity of antioxidant enzymes such as SOD, GPx, CAT, GST, and GR (Shearer et al., 1997). Therefore, there is an increase of free radical species in the aqueous humor and a signif-
ificant reduction in nicotinamide adenine dinucleotide phosphate (reduced form) and GSH content in lens (Bhuyan et al., 1981). Human senile cataract is also associated with a progressive loss of GSH; GSH is an important component of the lens because it protects the lens from oxidative damage and maintains the functions and transparency of the lens by protecting protein sulphydrol groups from oxidation (Harding, 1970). Various synthetic compounds and natural resources that are rich in antioxidants have been reported to prevent cataractogenesis in animal models (Tamada et al., 2001; Gupta et al., 2003, 2005; Doganay et al., 2006; Geraldine et al., 2006).

Ellagic acid, a naturally-occurring polyphenolic compound, is endowed with antimutagenic, anti-inflammatory, antiviral, antitumorigenic, and antioxidant activities. Previous studies have indicated that ellagic acid possesses scavenging action against both oxygen and hydroxyl radicals, and inhibits lipid peroxidation and 8-OhdG formations in vitro and in vivo (Cozzi et al., 1995; Takagi et al., 1995; Laranjinha et al., 1996; Iino et al., 2001). Although ellagic acid exhibits minimum solubility in water, it is highly soluble in organic sol-vents such as methanol and DMSO. This lipophilic property, along with its ability to scavenge peroxyl radicals, suggests that ellagic acid is a lipophilic, chain-breaking antioxidant.

In the present study, the mean activities of CAT, SOD, GPx and GST were found to be significantly lower in lenses of Group II (cataract-untreated) rats than those of Group I (normal) rats (Table 2). Other workers have made similar observations in cataractous lenses (Bhuyan and Bhuyan, 1977; Varma et al., 1982, 1984; Fecundo and Augusteyn, 1983; Dwivedi and Pratap, 1987; Cekic et al., 1999; Gupta et al., 2002). However, we observed that the mean activities of CAT and GPx in Group III lenses (treated with ellagic acid in selenite-challenged pups) were significantly higher than the values in Group II lenses (although still lower than the values in Group I lenses) (Table 2). Majid et al. (1991) observed an increase in the level of Gpx and GR in liver and lungs of ellagic acid-treated mice; similarly, Gpx was also found to be increased in regions of the brain (Hassoun et al., 2004). Our results suggest that ellagic acid can partially prevent the reduction in lenticular antioxidant enzyme activi-ties brought by exposure of rat pups to selenite; in our study, this protective effect was manifested morphologically as a de-
creased frequency and intensity of lenticular opacification (Table 1, Figs. 5—7). The higher mean activities of the antiox-idant enzymes seen in Group III rat lenses (ellagic acid-treated group) compared to Group II rat lenses (injected with selenite alone) highlight the putative anti-radical and antioxidant ef-fects of ellagic acid (Priyadarshini et al., 2002).

Selenite is known to cause a significant depletion of GSH and to increase damage to membranes, the latter being indi-cated by increased levels of MDA (Gupta et al., 2002). We ob-served that ellagic acid prevented cataract formation in at least half of the eyes in Group III rats (cataract-treated); in the eyes.

Fig. 8. Calcium level in lenses analyzed on postpartum day 30. Values are expressed as mean ($n = 5$) ± SD.
in which there were cataractous changes, the degree of opacification was less intense than that seen in Group II rats (Table 1). Lenses in Group III rats had higher mean GSH levels and lower mean MDA concentration than did Group II rats. These data suggest that the putative anticahtaractogenic effect of ellagic acid, as manifested in the gross morphological study, may also have occurred due to an increase in the GSH level and decrease in the MDA concentration.

In the present study, ellagic acid may have prevented or retarded oxidative damage to sulphhydril groups in the lens epithelium (the initial event in selenite cataractogenesis) by direct radical scavenging activity (Priyadarshini et al., 2002). Ellagic acid may also have indirectly reduced selenite-induced oxidative stress by its ability to restore GSH levels to normal; reduced GSH is believed to play a significant role in the maintenance of the reduced state in the lens. The GSH concentration has been found to decrease following selenite-induced cataract formation, and loss of reduced GSH from the nuclear region of the lens is believed to be the crucial feature that precedes age-related cataract formation (Giblin et al., 1976). Our observation that there was decreased frequency of cataract formation, associated with elevated activities of GSH and Gpx enzymes in lenses, in ellagic acid-treated Group III rats, is consistent with the results of a previous study in which elevated GSH and Gpx levels were noted in the liver of animals treated with ellagic acid (Majid et al., 1991). Ellagic acid has been shown to regulate intracellular GSH levels in brain tissues of mice by induction of gamma-glutamylcysteine synthetase (Carlson et al., 2003), and to prevent n-nitrosodiethyamine-induced lung tumorigenesis in mice by increasing the levels of GSH (Khanduja et al., 1999). The scavenging action of ellagic acid on both oxygen and hydroxyl radicals, and inhibition of lipid peroxidation and 8-OhdG formation in vitro and in vivo, has also been documented (Cozzi et al., 1995; Takagi et al., 1995; Laranjinha et al., 1996; Iino et al., 2001). The protective effects of ellagic acid can thus be attributed to several factors including binding of DNA, inhibition of the production of ROS, scavenging of ROS, and protection of DNA from alkylating injury.

From a study of the structure-function relationship, it has been suggested that both phenolic hydroxy groups and the lactone are necessary for the activity of ellagic acid at different conditions (Barch et al., 1996); these mechanisms are likely to have been operative in retarding the initial selenite-induced oxidative damage to lens membranes in the present study. Lens membrane phospholipids are known to be affected by oxidative stress, with a consequent increase in lipid peroxidation, which is manifested by a rise in the level of malondialdehyde (Varma et al., 1979, 1995; Varma and Hegde, 2004). The efficacy of ellagic acid in inhibiting lipid peroxidation has been well-documented in rat liver microsomes (Priyadarshini et al., 2002), N-nitrosodiethyamine (NDEA)-induced lung tumorigenesis (Khanduja et al., 1999) and in hyperlipidemic rabbit serum (Yu et al., 2005). This property of inhibiting lipid peroxidation possibly accounted for the observed decline in malondialdehyde level in the present study in selenite-challenged rats that received ellagic acid.

In conclusion, we have demonstrated, for the first time, the prevention of selenite-induced cataractogenesis in Wistar rats by administration of ellagic acid. This effect is associated with increased GSH, maintaining of antioxidant enzyme activities and decreased MDA levels. These biochemical changes reiterate the important role of oxidative stress in selenite-induced cataractogenesis, with ellagic acid playing the role of antioxidant. Our preliminary results are encouraging, but further molecular studies are needed to clarify the mechanism behind the anticahtaractogenic effect of this compound.

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Alterations in lenticular proteins during ageing and selenite-induced cataractogenesis in Wistar rats

Muniyan Sakthivel,1 Rajan Elanchezhian,1 Philip A. Thomas,2 Pitchairaj Geraldine1

1Department of Animal Science, School of Life Sciences, Bharathidasan University, Tamil Nadu, India; 2Institute of Ophthalmology, Joseph Eye Hospital, Tamil Nadu, India

Purpose: To determine putative alterations in the major lenticular proteins in Wistar rats of different ages and to compare these alterations with those occurring in rats with selenite-induced cataract.

Methods: Lenticular transparency was determined by morphological examination using slit-lamp biomicroscopy. Alterations in lenticular protein were determined by sodium dodecyl sulfate-PAGE (SDS-PAGE) and confirmed immunologically by western blot.

Results: Morphological examination did not reveal observable opacities in the lenses of the rats of different age groups; however, dense nuclear opacities were noted in lenses of rats in the selenite-cataract group. Western blot assays revealed age-related changes in soluble and urea-soluble lenticular proteins. Decreased αA- and βB1-crystallins in the soluble fraction and aggregation of αA-crystallin, in addition to the degraded fragment of βB1-crystallin, in the urea-soluble fraction appeared to occur in relation to increasing age of the rats from which the lenses were taken; similarly, cytoskeletal proteins appeared to decline with increasing age. The lenses from rats in the selenite-cataract group exhibited similar changes, except that there was also high molecular weight aggregation of αA-crystallin.

Conclusions: The results of this study suggest that there is loss, as well as aggregation, of αA-crystallin in the aging rat lens, although there is no accompanying loss of lenticular transparency.

Over the last twenty years, the causes of blindness have changed in proportion and actual number. However, cataract has remained the major cause of blindness globally, particularly in Asia [1]. Cataract is a multifactorial disease associated with several risk factors such as aging, diabetes, exposure to sunlight, and hypertension; however, free radical-induced oxidative stress is postulated to be, perhaps, the major factor leading to senile cataract formation [2]. Generation of reactive oxygen species (ROS), resulting in degradation, crosslinking, and aggregation of lens proteins, is regarded as an important factor in cataractogenesis [3-5].

The mammalian lens consists mainly of proteins, which account for over 30% of its weight [6]. It is the architecture of the crystalline lattice that is ultimately responsible for lenticular transparency and for the proper focusing of light. The highly concentrated cellular proteins in mammalian lenses consist of two families of water-soluble lens crystallins, namely, α-crystallin and β/γ-crystallin [7], that are uniformly distributed in transparent lenticular cells. Dense opacification results when the proteins form large insoluble aggregates that approach or exceed the dimensions of the wavelength of light and produce large fluctuations in the index of refraction that result in increased light scattering [3,6,8].

Numerous changes in crystallin structure occur with age, mainly due to post-translational, non-enzymatic reactions [9]. Protein synthesis and turnover predominantly occur in the cortical region, and decrease with age in the nucleus. Previous experimental studies have shown the importance of cytoskeletal and membrane-associated proteins in the development and maintenance of the transparent structure of the cells of the lens [10-14]. Cytoskeletal proteins constitute 2%-4% of lenticular proteins, which include intermediate filaments, microfilaments, and microtubules [15,16]. Although the major proteins of the cytoskeleton of the lens are actin and vimentin, the tissue is also known to contain several similar proteins including tubulin, ankyrin, α-actinin, spectrin, myosin, tropomyosin, and beaded filaments [15-17]. Typically, the concentration of cytoskeletal proteins in the lens decreases with age, particularly in the nucleus. Loss of these proteins, including vimentin and α-actinin, is accelerated in the human lens during the development of senile cataract [12,18]. Therefore, studies of the protein patterns in the lens are of considerable importance with respect to an understanding of changes associated with aging and cataractogenesis.

Different animal models have been developed with a view to better understand the aging process and cataract formation [19-22]. None of these models exactly matches what occurs in human cataractogenesis. However, the model of selenite-induced cataractogenesis has many features to recommend it, namely: the morphological and biochemical characteristics have been extensively investigated; this model
shows several general similarities to human cataract; the selenite cataract is rapidly induced; it is a useful rodent model for rapid screening of potential anticitaract agents [23]. The aim of the present study was to determine the occurrence of possible alterations in certain crucial lenticular proteins (αA- and βB1-crystallins, and cytoskeletal proteins) during the process of aging, and to compare these changes with those occurring in selenite cataracts in Wistar rats.

**METHODS**

**Experimental animals:** Rats of the Wistar strain were used in this study. The rats were housed with parents in large spacious cages, and the parents were given food and water ad libitum. The animal room was well ventilated and had a regular 12:12 h light/dark cycle throughout the experimental period. These animals were used in accordance with institutional guidelines and with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Research. Rats in the following age groups were randomly selected (5 animals in each age group): 10-day-old, 16-day-old, 30-day-old, 90-day-old, 180-day-old, 365-day-old rats. Sixteen-day-old rats with selenite-induced cataract were also studied.

**Selenite cataract:** The cataractogenesis was induced by a single subcutaneous injection of sodium selenite (19 μmoles/kg bodyweight) on postpartum day 10.

**Morphological observations:** A slit-lamp biomicroscopic examination of each eye of 16-day-old and 365-day-old rats was performed to determine possible lenticular opacification (morphological assessment). Prior to performing the examination, mydriasis was achieved by using a topical opthalmic solution containing tropicamide with phenylephrine (Maxdil Plus; Hi-Care Pharma, Chennai, India); one drop of the solution was instilled every 30 min for 2 h into each eye of each rat, with the animals being kept in a dark room, and, after 2 h, the eyes were viewed by the slit-lamp biomicroscope at 12× magnification.

The animals were sacrificed by cervical dislocation; the eyes of the different age groups were collected and the lenses were removed. Each lens was then homogenized with 10 times its mass of 20 mM phosphate buffer containing 1 mM ethylene glycol-bis(2-aminohexanate)-N,N,N’,N’-tetraacetic acid (EGTA; pH 7.2), and centrifuged at 14,000× g at 4 °C for 15 min. This process was repeated twice. The supernatant obtained was used for the determination of waster soluble protein (WS) while the pellet dissolved in 8 M urea was considered as urea-soluble (insoluble) protein. The protein concentration in each sample was estimated by the method of Bradford [24]. The estimated value of protein in each sample was used to calculate the ratio of urea-soluble to soluble protein. Both the fractions (soluble and urea-soluble) were aliquoted into portions and stored at −70 °C until analyzed by immunoblot.

**Immunoblot:** Immunoblot analysis was performed to determine the relative concentrations of the cytoplasmic proteins, αA-crystallin and βB1-crystallin, and of the cytoskeletal proteins, actin, vimentin, and spectrin, in samples of the various age groups. Proteins subjected to SDS–PAGE were electrophoretically transferred to a nitrocellulose (NC) sheet using a semidyry blotting apparatus (Bio-Rad, Richmond, CA). Blotting was done at 25 V for 2 h. Blotted membranes were stained by Ponceau S solution to check for the efficiency of transfer; subsequently, blocking was done with 5% non-fat dry milk in Tris buffer saline (pH 7.5) with 0.1% (v/v) Tween-20 for 3 h. Antibodies (purchased from Sigma, St. Louis, MO) against αA-crystallin (1:1,000 dilution), βB1-crystallin (1:2,000 dilution), actin (1:100 dilution), vimentin (1:50 dilution) and spectrin (1:400 dilution) were used. Immunoreactivity was visualized with alkaline phosphatase conjugated to anti-mouse IgG or anti-rabbit IgG secondary antibodies and 5-bromo 4-chloro 3-indolyolphosphate/nitroblue tetrazolium chloride (BCIP/NBT; Genei, Bangalore, India). To analyze the small changes observed in the intensity of the bands, densitometry was conducted on the scanned images of the membrane. The program Quantity One SW (Bio-Rad) was used for the analysis of intensity of bands in each lane of the membrane.

**Statistical analysis:** Statistical analysis was performed with SPSS software (version 11.0) (SPSS Corporation, Chicago, IL). The results are expressed as the median (min–max), as the non-parametrical distribution requires nonparametrical analysis that compares medians instead of means. To test for correlations between two parameters, Spearman analysis was performed. P-values <0.05 were considered statistically significant.

**RESULTS**

**Morphological examination and protein ratio:** Morphological examination revealed clear transparent lenses in both eyes in all 10-day-old and 365-day-old rats. However, a complete nuclear opacity was observed in the lenses of each eye in 16-day-old rats that had been administered selenite (Figure 1). The ratio of urea-soluble to soluble lenticular protein was found to rise with increasing age from 0.027 (10-day-old rats) to 0.126 (365-day-old rats) and was found to be 0.127 in cataractous lenses (16-day-old, selenite-administered rats; Figure 2).

**Immunoblot of αA- and βB1-crystallin:** The relative amounts of αA- and βB1-crystallin in the soluble and urea-soluble fractions were further examined by immunoblotting with specific antibodies. Immunoblot analysis revealed that there was a decrease of αA-crystallin in the soluble fraction with increasing age, and this decrease was also seen in lenses with selenite cataract (Figure 3). There was also an obvious decrease of αA-crystallin in the urea-soluble fraction with increasing age; however, the anti-αA-crystallin antibody
appeared to detect appreciable amounts of polymeric species of αA-crystallin with increasing age; such polymeric species of αA-crystallin were not detected in the soluble fraction or in lenses with selenite cataract (Figure 4). We also performed Spearman correlation analysis to examine whether intensity levels of αA-crystallin correlated well with the urea-soluble to soluble protein ratio. Figure 3C and Figure 4C demonstrate that there was a significant negative correlation (r=-0.890, p<0.01 and r=-0.941; p<0.01) between the band intensity levels of αA-crystallin and urea-soluble to soluble protein ratio in soluble and urea-soluble protein fractions, respectively.

Figure 1. Appearance of lenses in different groups of Wistar rats. Morphological assessment of cataract formation in Wistar rat pups by the slit-lamp biomicroscope. Clear lens observed in 16 day-old rats (A) and 365 day-old rats (B), and dense opacification of the lens observed in 16 day-old, selenite-administered rats (C).

Figure 2. Effect of aging on the ratio of urea-soluble to soluble protein in lenses of rats of different age groups in comparison to rats with selenite cataractous lenses. The X-axis indicates the age in days and the Y-axis indicates the ratio of urea-soluble to soluble protein. 10 =10 day-old rat lenses; 16 = 16 day-old rat lenses; 30 = 30 day-old rat lenses; 90 = 90 day-old rat lenses; 180 = 180 day-old rat lenses; 365 = 365 day-old rat lenses; 16(se) = lenses of 16 day-old rats that had been administered selenite on post-partum day 10. The ratio of urea-soluble to soluble lenticular protein was found to rise with increasing age.

Figure 3. Occurrence of αA-crystallin in lenticular soluble protein fractions in different groups of Wistar rats. A typical western blot showing age-dependent relative concentrations of αA-crystallin in the soluble protein fractions of Wistar rat lenses. A: An equal amount of protein was loaded in each lane and subjected to electrophoresis. Blots were incubated with αA-crystallin antibody, with β-actin as the loading control. B: band intensity of αA-crystallin levels relative to 10 day-old rat lenses. C: Linear regression analysis revealed a significant negative correlation between αA-crystallin and the urea-soluble to soluble protein ratio. (▲) Values in 10 day-old rat lenses; (□) Values in 16 day-old rat lenses; (●) Values in 30 day-old rat lenses; (●) Values in 90 day-old rat lenses; (×) Values in 180 day-old rat lenses; (■) Values in 365 day-old rat lenses; (○) Values in the lenses of 16 day-old rats that had been administered selenite on post-partum day 10.
Similarly, the relative amount of βB1-crystallin in the soluble fractions was also found to decrease with increasing age (Figure 5). When the urea-soluble fraction was also examined for the presence of βB1-crystallin, decreased band intensity with a small fragmented band was observed in the lenses of aged rats, compared to the pattern in 10-day-old rats; in selenite-cataractous rat lenses, the fragment was seen at different places (Figure 6). Spearman analysis of correlation was also performed to examine whether there was any correlation between the intensity of the band corresponding to βB1-crystallin and the ratio of urea-soluble to soluble proteins. Figure 5C and Figure 6C demonstrate that there was a significant negative correlation between the intensity of the band corresponding to βB1-crystallin and the ratio of urea-soluble to soluble proteins in both the soluble ($r=0.923$; $p<0.01$) and urea-soluble ($r=0.877$; $p<0.01$) fractions.

**Immunoblot of cytoskeletal proteins:** To identify the high molecular weight cytoskeletal proteins which were altered with increasing age, and in selenite cataract lenses, western blot analyses were performed on the soluble and urea-soluble protein fractions of the lenses of all groups. But no differences were found in the soluble fraction for high molecular weight proteins, actin and vimentin, in all the measured groups (Figure 7). In the urea-soluble fractions, the 42 kDa band reacted with anti-actin, the 57 kDa reacted with monoclonal anti-vimentin and the 235 kDa band reacted with polyclonal anti-spectrin (Figure 8).

Quantitation of proteins was performed by densitometry. When compared to the values obtained in lenses of 10-day-old rats, a gradual decrease in the concentrations of these identified proteins was noted with increase in the ages of the test rats (10 days to 365 days) and this correlated with urea-soluble to soluble protein ratio for actin ($r=0.867$, $p<0.01$), vimentin ($r=-0.933$, $p<0.01$) and spectrin ($r=0.822$, $p<0.01$).

Considering the levels of these proteins to be 100% on day 10, the levels of actin, vimentin and spectrin were found to be decreased to 53%, 51%, and 12%, respectively, in the lenses with selenite-induced cataract (Figure 8).

**DISCUSSION**

The proteins that constitute the innermost core of the nucleus of the lens may be older than the postnatal life of the animal. Due to minimal or no turnover, lenticular proteins are subjected to considerable post-translational modifications which, in turn, disrupt lenticular architecture and alter the optical properties, leading to formation of a senile cataract [9]. We sought to determine the extent of age-related alterations in lenticular proteins in Wistar rats, and to compare these with the changes occurring in selenite-induced cataractous lenses.

In the present study, the ratio of urea-soluble to soluble lenticular proteins was found to increase with increasing age of the animals from which the lenses were taken (Figure 2), suggesting age-related increase in insolubilization of these proteins. Swamy and Abraham [25] observed that in rats, there was an increase in soluble proteins with age up to 20 months and a slow decrease in the later ages whereas urea-soluble proteins showed a continuous increase during the experimental period.
Age-related changes have been documented in the protein αA-crystallin, which is found abundantly in the lens; the molecular chaperone properties of this protein are believed to play a key role in maintaining transparency of the lens [26, 27]. Changes in the αA-crystallin molecule possibly influence the lenticular changes that occur during normal development and aging [9,28-30]. With aging, αA-crystallin is believed to bind partially unfolded lenticular β- and γ-crystallins, thereby preventing their aggregation and precipitation and therein delaying lenticular opacification [31]. In the present investigation, an interesting observation made was the loss of αA-crystallin (20 kDa) in the soluble protein fraction and a gradual loss in the intensity of the band in the urea-soluble fraction with increasing age of the animals from which the lenses were taken (Figure 3 and Figure 4). Relative to αA-crystallin from 10 day-old rat lenses, anti-αA-crystallin antibody could detect an additional high molecular weight aggregate in the urea-soluble fraction (Figure 4).

It has been suggested that high molecular weight proteins might represent an intermediate stage in conversion of water-

![Figure 5](image1.png)

Figure 5. Occurrence of βB1-crystallin in lenticular soluble protein fractions in different groups of Wistar rats. A typical western blot showing age-dependent relative concentrations of βB1-crystallin in the soluble protein fractions of Wistar rat lenses. A: An equal amount of protein was loaded in each lane and subjected to electrophoresis. Blots were incubated with βB1-crystallin antibody. B: band intensity of βB1-crystallin levels relative to 10 day-old rat lenses. C: Linear regression analysis revealed a significant negative correlation between βB1-crystallin and the urea-soluble to soluble protein ratio. (▲) Values in 10 day-old rat lenses; (●) Values in 16 day-old rat lenses; (■) Values in 20 day-old rat lenses; (●●) Values in 20 day-old rat lenses; (▲▲) Values in 180 day-old rat lenses; (■■) Values in 365 day-old rat lenses; (○) Values in the lenses of 16 day-old rats that had been administered selenium on post-partum day 10.

![Figure 6](image2.png)

Figure 6. Occurrence of β1-crystallin in lenticular urea-soluble protein fractions in different groups of Wistar rats. A typical western blot showing age-dependent relative concentrations of β1-crystallin in the urea-soluble protein fractions of Wistar rat lenses. A: An equal amount of protein was loaded in each lane and subjected to electrophoresis. Blots were incubated with β1-crystallin antibody. B: Band intensity of β1-crystallin levels relative to 10 day-old rat lenses. C: Linear regression analysis revealed a significant negative correlation between βB1-crystallin and the urea-soluble to soluble protein ratio. (▲) Values in 10 day-old rat lenses; (●) Values in 16 day-old rat lenses; (■) Values in 20 day-old rat lenses; (●●) Values in 20 day-old rat lenses; (▲▲) Values in 180 day-old rat lenses; (■■) Values in 365 day-old rat lenses; (○) Values in the lenses of 16 day-old rats that had been administered selenium on post-partum day 10.
soluble to water-insoluble proteins in rabbit lenses [32]. Swamy and Abraham [25] showed that with increasing age, there was an increase of high molecular weight and insoluble proteins, with disappearance of γ-crystallins and sulfhydryl groups from the soluble fraction and an increase in the insoluble fraction and high molecular weight aggregates. Bindels et al. [33] proposed that in rodents, soluble high-molecular weight aggregates are formed by polymerization chiefly of α-crystallins.

Because of the possible function of βB1-crystallin in controlling the higher assembly of β-crystallins and the potential role of truncated versions of the protein in cataract formation [9], the present study also included an evaluation of the state of βB1-crystallin. In the present investigation, there appeared to be a loss of βB1-crystallin (30 kDa) in both soluble and urea-soluble fractions of lenticular protein with increasing age of the rats (Figure 5 and Figure 6). This finding is supported by previous studies that have shown that age-related proteolytic processing of human lenticular β-crystallins occurs mainly at the NH₂-terminal extensions [34-36], and that the first crystallins modified are βB1- and βA1/A3-crystallin [34-37]. Here again, the appearance of an additional faint band in the soluble fraction, and a new band of molecular weight less than 30 kDa in the urea-soluble fractions may have been due to the cleavage of NH₂-terminal extensions of βB1-crystallin. The presence of various NH₂-terminally truncated forms of βB1-crystallin in the lower-molecular-weight fractions, together with the extensive degradation (15-41 residues) of the NH₂-terminally truncated forms of βB1-crystallin with age as shown by mass spectrometry, strengthens the idea that the NH₂-terminal extension of βB1-crystallin is also involved in higher-order assembly [38].

Cytoskeletal proteins comprise 2%-4% of lenticular proteins and include actin, tubulin, vimentin, and spectrin. Cytoskeletal and membrane-associated proteins in the lens

Figure 7. Occurrence of high molecular weight protein in lenticular soluble protein fractions in different groups of Wistar rats. Detection of changes in high molecular weight soluble protein by western blot analysis. An equal amount of the soluble protein fractions were loaded in each lane and subjected to 4%-20% SDS-PAGE. 10 = 10 day-old rat lens and the standard M = Molecular weight marker (A). Blots were subjected to immunoblot detection. Immunoblot analysis did not reveal any differences in the fraction for high molecular weight proteins, vimentin (B) and α-actin (C), in all the groups measured.

Figure 8. Occurrence of high molecular weight proteins in lenticular soluble protein fractions in different groups of Wistar rats. Detection of changes in high molecular weight insoluble protein by western blot analysis. Panels A, D, and G show immunoreactive bands of spectrin (240 kDa), vimentin (57 kDa), and α-actin (42 kDa), respectively. Box-plots show the band intensity of spectrin, vimentin, and α-actin, relative to the level in 10 day-old rat lenses (panels B, E, and H, respectively). Linear regression analysis revealed a significant negative correlation between the urea-soluble to soluble protein ratio and the levels of spectrin, vimentin, and alpha-actin (panels C, F, and I, respectively). (A) Values in 10 day-old rat lenses; (B) Values in 16 day-old rat lenses; (C) Values in 30 day-old rat lenses; (D) Values in 90 day-old rat lenses; (E) Values in 180 day-old rat lenses; (F) Values in 365 day-old rat lenses; (G) Values in the lenses of 16 day-old rats that had been administered selenite on post partum day 10.
may have important functions in the development and maintenance of the transparent structure of the lens. In the present investigation, it was observed that with an increase in the ages of the rats from 10 days to 365 days, there was a 43% loss of vimentin, 68% loss of actin and 88% loss of spectrin in the lenses sampled. Immunoblot analysis did not reveal any variation in the relative quantity of actin and vimentin in the soluble fraction (Figure 7). Interestingly none of the changes was associated with loss of lenticular transparency as assessed morphologically (Figure 1). It has previously been reported that the age-related loss of vimentin, tubulin, and other cytoskeletal proteins in the nucleus of the human lens is not a direct initiator of nuclear cataract, since the same changes are evident even in old, clear (non-opaque) lenses [39-42].

When we examined the profile of lenticular proteins in selenite-induced cataractous lenses from 16-day-old rat pups, we observed changes that were very similar to the age-related changes occurring in rats ranging from 16-days-old to 365-days-old (Figure 2). There was an increase in the ratio of ureasoluble to soluble proteins (Figure 2) and the formation of insoluble protein aggregates of high molecular weight (Figure 2). Immunoblot analysis also revealed a decreased intensity of the 30 kDa β1-crystallin in selenite-induced cataractous lenses (compared to 16-day-old [age-matched] lenses); such a decrease has been previously reported [43-45]. Immunoblot analysis also revealed a decrease of a αA-crystallin in the soluble fraction of lenses with selenite cataract (Figure 3), although polymeric species of a α-crystallin were not detected in these lenses (Figure 4). With respect to cytoskeletal proteins, actin, vimentin, and spectrin were found to be decreased by as much as 50% to 88%, and proteins of molecular weight 42, 57, and 235 kDa were also markedly reduced. Matsushima et al. [45] also noted a similar reduction of these proteins to non-detectable levels in the nucleus and to 40% in the cortex of selenite cataracts. Loss of lenticular cytoskeletal proteins has been reported in oxidative stress-induced cataract models using buthionine sulfoxamine and selenite, with calcium activated proteolysis of calpain believed to be the major cause of the protein loss [45,46]. Hyperbaric oxygen-induced opacification of guinea pig lenses has also been reported to result in loss of cytoskeletal proteins due to oxidative damage resulting in disulfide cross-linkage, leading to high molecular weight protein aggregation [47]; loss of cytoskeletal protein due to high molecular weight protein aggregates have also been noted in human lenses [40,41].

Since similar changes occurred in the profile of lenticular proteins in rats of increasing age (up to 365 days) and in rats with selenite-induced cataract, the question arises as to why no lenticular opacification was noted in rats ranging in age from 10 days to 365 days, whereas dense nuclear opacification was only noted in rats that had been administered selenite. Possibly, an increased quantity of insoluble protein alone is not sufficient to cause lenticular opacification; it has been observed that some older, normal human lenses have a greater amount of water-insoluble protein than some younger, cataractous lenses [3]. Lenticular opacification also probably involves not simply quantitative, but also qualitative, changes related to the refractive index. Dense opacification results when the proteins form large insoluble aggregates that approach or exceed the dimensions of the wavelength of light and produce large fluctuations in the index of refraction that result in increased scattering of light [3,6,8]. In addition, there may be other, unique, factors that contribute to maintenance of transparency of the lens even when insolubilization of lenticular proteins has occurred. Elucidation of these factors may contribute to a better understanding of the mechanisms involved in cataractogenesis, and lead to improved methods of preventing or delaying the onset of cataract formation.

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